cDNA Labeling with Aminoallyl- dUTP (& Ambion buffers)

A. Probe labeling and purification

1. Remove all reagents required for each reaction from the freezer. Place the enzyme solutions on ice immediately. Thaw the other reagents at 37ºC for ~ 5 minutes mix and store on ice until use (except the water, which should be stored at room temperature).

2. Combine the following to a final volume of 15.5 µl

Reagent	Volume(µl)
Primer P2	2.0 (2ug/ul)
Total RNA	(1-10 ug)
RNasin (inhib)	1.0
DEPC water	To 15.5 ul

Heat the mixture at 70°C for 10 min, then place on ice to chill for 10 minutes. Briefly centrifuge to collect all components to the bottom of the tube.

3. Add the following reagents in the order listed (if desired, a stock can be made up that will support several labeling reactions):

REAGENT	Volume(µl)
5X buffer	6.0
50X aa dUTP/dNTPs	0.6
DTT (0.1 M)	3.0
SuperScript RT	1.9
DEPC water	3.0
Total	**14.5**

50X aa dUTP/dNTPs: 10 ul each of dATP (100 mM), dGTP (100mM), and dCTP (100mM); 4 ul of aa dUTP (100 mM), 6 ul of dTTP (100mM).

4. Pipet up and down gently to thoroughly mix the reaction mixture and incubate at 42 °C for 2 hour

5. Briefly centrifuge the reaction and add 10µl of 0.5M EDTA to each reaction mixture to stop the reaction. (Note: you can interrupt the procedure at this point and keep the reaction mixture at 4ºC overnight).

6. Add 10µl of 1M NaOH to each reaction mixture and mix.

7. Incubate at 65ºC for 30 minutes.

8. Neutralize with 10µl of 1M HCl.

9. Concentrate and purify cDNA using Qiagen MinElute PCR Purification Kit:

9-1. Fill the microcentrifuge tube (1.5 ml) with 300 µl Buffer PB.
9-2. Add 60 µl of the neutralized reaction to Buffer PB.
9-3. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
9-4. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min. For maximum recovery, transfer all traces of sample to the column.
9-5. Pour the flow-through back onto the top of the column and centrifuge again for 1 min.
9-6. Discard flow-through. Place the MinElute column back into the same tube.
9-7. To wash, add 750 µl Buffer PE to the MinElute column, incubate for 5 min at room temperature and centrifuge for 1 min.
9-8. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1min at maximum speed.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
9-9. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.
9-10. To elute DNA, add 10 µl of H_2O (pH between 7.0 and 8.5) to the center of the membrane, let the column stand for 1 min, and then centrifuge for 5 min.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.
The average eluate volume is 9 µl from 10 µl elution buffer volume.
9-11. Repeat step 9-10 two more times, collecting 27µl of purified cDNA .
(Note: You can interrupt the procedure at this point and keep the reaction mixture at -20ºC overnight).
9-12. Add 3µl of 1M sodium bicarbonate (pH 9.3) to the cDNA solution.
Add cDNA solution to the dye, if dye has been aliquioted and dried in a SpeedVac; or add 1-2 µl of the dye directly to cDNA solution, if the dye was resuspended in 16µl DMF or DMSO.
9-13. Mix the dye and cDNA; incubate at RT for 1-2 hours in a dark on the shaker (60 rpm)

10. Stop the labelling reaction by adding 4.5µl of 4M hydroxylamine hydrochloride to the reaction mixture. Mix and briefly centrifuge.

Incubate at RT for 30 min in the dark.

11. To remove unicorporated/quenched cye-dyes proceed with QIAGEN Qia-quick PCR Purification Kit (11) or QIAGEN MinElute Purification Kit (12):

Qia-quick PCR purification kit:

11-1. Combine Cy3 and Cy5 reactions.
11-2. Add 30µl of water to the mixture.
11-3. Add 500 µl of Buffer PB to the mixture of labelled cDNAs.
11-4. Place a QIAquick spin column in a provided 2 ml collection tube.
11-5. Apply the sample to the QIAquick column and centrifuge at 13,000 rpm for 30-60 sec.

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11-6. Discard flow-through. Place the QIAquick column back into the same tube.
11-7. To wash, add 750µl Buffer PE to the QIAquick column and centrifuge for 30-60 sec.
11-8. Discard flow-through and place the QIAquick column back into the same tube.
11-9. Repeat step 11-7.
11-10. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1min at maximum speed.
11-11. Place QIAquick column in a clean 1.5 ml microfuge tube
11-12. To elute Cy-labelled cDNA, add 30µl of EB buffer or ultra pure water to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge at 13,000 rpm for 1 min.
11-13. Repeat elution step one more time.

MinElute Purification Kit:

12-1. Combine Cy3 and Cy5 reactions.
12-2. Add 30µl of water to the mixture.
12-3. Add 500 µl of Buffer PB to the mixture of labelled cDNAs.
12-4. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
12-5. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min. *For maximum recovery, transfer all traces of sample to the column.*
12-6. Discard flow-through. Place the MinElute column back into the same tube.
12-7. To wash, add 750 µl Buffer PE to the MinElute column, incubate for 5 min at room temperature and centrifuge for 1 min.
12-8. Discard flow-through and place the MinElute column back in the same tube. Centrifuge the column for an additional 1min at maximum speed.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12-9. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.
12-10. To elute DNA, add 10 µl of Buffer EB to the center of the membrane, let the column stand for 1 min, and then centrifuge for 5 min.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.
The average eluate volume is 9 µl from 10 µl elution buffer volume.

B. Hybridization

Here we used Ambion SlideHyb Glass Hybridization Buffers. What follows is described as used for our particular arrays. Note that the Ambion SlideHyb Survey Kit (Cat # 1860) is designed to allow users to determine the best conditions to use for hybridization, which may differ from ours, below. Users may find a different buffer and/or temperature is better suited for their arrays.

1. Preheat SlideHyb Buffer #4 (or the best buffer for the array used) to 68°C for 15–30 minutes. Swirl the SlideHyb Buffer until all precipitated material has dissolved. The SlideHyb Buffers remain stable with repeated heating to 68°C, so the entire contents of the tubes can be pre-warmed to 68°C before removing an aliquot for hybridization.

2. Heat denature the labeled cDNA at 95°C for 10 mins. Centrifuge to collect the liquid at the bottom of the tube.

3. Add warm SlideHyb Buffer to labeled cDNA. The ratio 1:10 (labeled cDNA : hybridization buffer) should be kept.

4. Apply the SlideHyb/cDNA mixture to the microarray (add 2x12ul of 3xSSC to the chamber) and hybridize overnight (14–24 hrs.) in a humidity chamber at 50°C (or the best temperature as determined beforehand using the SlideHyb Survey Kit).

C. Wash off unbound fluorescent cDNA

1. Prepare low and high stringency wash buffers using 20X SSC and 10% SDS as follows:

1-1. Low stringency wash buffer
Final concentration for 500 ml:

2X SSC (50 ml of 20X SSC)
0.5% SDS (25 ml of 10% SDS)
water to 500 ml

1-2. High stringency wash buffer
Final concentration for 500 ml:

0.5X SSC (12.5 ml of 20X SSC)
0.5% SDS (25 ml of 10% SDS)
water to 500 ml

2. Preheat the wash buffers to the temperature used for hybridization.

3. Wash the microarray 2 x 15 minutes in 50 ml of low stringency wash buffer at the temperature used for hybridization.

4. Wash the microarray 2 x 15 minutes in 50 ml of high stringency wash buffer at the temperature used for hybridization.

5. Transfer the slide to a slide rack and centrifuge at low rpm (700-1000) for 5 minutes in a clinical centrifuge equipped with a horizontal rotor for microtiter plates.

If the slide is simply air dried, it frequently acquires a fluorescent haze. Centrifuging off the liquids results in a lower fluorescent background. As the rate of drying can be quite rapid, it is suggested that the slide be placed in the centrifuge immediately upon removal from the Coplin jar.